Biacore™ systems in discovery and early-stage development of biotherapeutic antibodies
Abstract
Advances in the use of antibodies for the treatment of disease has driven demand for new antibody formats designed to improve the efficacy of the therapy and to reach new targets. Biacore systems are extensively used in biotherapeutic antibody discovery and development and here we discuss their utility at all stages of the process from selection of first candidates to clinical lead. We show that a combination of Biacore instruments, software, sensor chips, and kits supports the setup of screening and characterization assays and reduces assay development efforts. In screening, antibody capture followed by an antigen injection permits selection of monophasic and stable binders with the preferred kinetics and stoichiometry. Standardized epitope binning procedures ensure reliable determination of epitope specificity and the effect of antibody engineering efforts can be investigated by analysis of antibody binding to antigen and Fcγ-receptors. Developability aspects are also addressed, for instance by ensuring critical binding properties remain unchanged in forced degradation studies. A further advantage of using Biacore is that antibody concentration and kinetics can be monitored in the presence of nonbinding unfolded fractions, host cell proteins, and other impurities.

Introduction
Over the last 30 years, recombinant proteins including hormones, cytokines, and therapeutic antibodies have been developed for treatment of a variety of diseases including diabetes, cancer, and rheumatic disorders. Recombinant insulin in various forms is probably the most prescribed biotherapeutic medicine and while hormones and cytokines represent an important class of biotherapeutics, antibodies are now much more in focus and will eventually have a wider applicability. The number of FDA approved antibodies is steadily increasing (1) and while several of these antibodies are directed against the same target molecule, the target space is growing with approved antibodies now directed to over thirty different target molecules. By extrapolating the current approval trend* the number of approved antibodies (excluding biosimilars) is likely to double within the next ten years (Fig 1).

New antibody formats
A large majority of the approved antibodies are full-length antibodies and are of IgG1, IgG2, or IgG4 subclass. However, non-traditional antibody formats are slowly emerging. For example, blinatumomab (Blincyto™, approved 2014) is a bispecific antibody while brentuximab vedotin (Adcetris™, approved 2011) and ado-trastuzumab emtansine (Kadcyla™, approved 2013) are based on conventional antibodies but are conjugated with cytotoxic agents and are so-called antibody-drug conjugates. New formats are introduced to improve the efficacy (2) of the therapies and to reach new targets, for instance by designing antibodies that have the capability to cross the blood-brain barrier (3). Antibody formats that retain the basic structure of IgGs may inherit their pharmacokinetic properties (4) but novel constructs that lack the Fc-part of the antibody may have reduced half-life (5). This can be an advantage if the antibody is used for imaging purposes (6). However, for therapeutic purposes, there needs to be a balance between efficacy and half-life for smaller antibody formats such as single chain Fvs and nanobodies or other scaffolds that have the potential to reach more hidden targets and even to act as intrabodies to target intracellular antigens (7). Several pharmaceutical companies now have bispecific antibodies (8) and antibody-drug conjugates in their clinical pipeline (9) while intracellular antibodies may still be in research phase.

From antibody generation to clinical lead
The antibody development workflow (Fig 2) has evolved and early development is no longer focused entirely on potency and functional aspects such as specificity, affinity, and kinetics for their molecular targets. While these factors are crucial, developability aspects play an increasingly important role for reducing the risk of a later failure of the development program (10).

Developability aspects include studies on:
- The impact of post-translational modifications on stability and conformation (11, 12).
- Aggregation and fragmentation tendencies (13).
- Solubility and solution stability (14).
- Biological factors such as immunogenicity (15) and pharmacokinetic properties (16).

Developability studies thus try to answer questions such as: Can the antibody be manufactured? Is it safe? Will it have acceptable bioavailability and efficacy?

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* Extrapolation based on fitting the existing data to linear, polynomial, power, exponential, and logarithmic functions and presenting best fit data.
Efficiently select and optimize antibody candidates

Biacore was incorporated in the antibody development workflow almost immediately after the launch of the first Biacore system in 1990 when kinetic analysis of antibody-antigen interactions (17) and epitope binning procedures (18) were described. Antibody D2E7, that was later to become Humira, the bestselling drug in 2014 (19), was selected from a Biacore screen in the mid-1990s (20). From screening of candidates, through antibody engineering and into final development, Biacore systems have consistently been used to determine specificity of binding to characterize antibody-antigen and antibody-Fc receptor interactions and to guide development towards a clinical lead. In developability studies Biacore systems are typically used to monitor effects of forced degradation on antigen and Fc gamma receptor binding and for assessment of pharmacokinetic properties where binding to FcRn is related to antibody half-life. More recently the use of binding mode-specific reagents (21) has been described for detection of changes in antibody topography as a consequence of forced degradation.

Biacore systems in antibody development

Depending on the design of the flow system, direct interaction analysis with several target molecules can be performed with a single injection of sample. Typically, one measuring spot is used for active analysis and one for referencing (Fig 3). Depending on the Biacore system, from 1 to 8 active/reference pairs can be used. Independently of the number of active reference pairs, double referencing is typically performed to arrive at high quality data by subtracting blank cycles.

Minimize time spent on assay development with dedicated sensor chips and reagents

Antibody applications are supported by a number of sensor chips and reagents provided with ready-to-use protocols enabling rapid assay development using well known and reversible capture formats.
Biacore Sensor Chip Protein A (Fig 4A) can be used directly for antibody concentration measurements or for capture of antibodies and subsequent kinetic analysis of antibody–antigen interactions. Biacore Sensor Chip CM5 (Fig 4B) can be used for direct immobilization and can be combined with several antibody capture kits. With the Mouse Antibody Capture Kit, all IgG subclasses and IgM and IgA can be captured to immobilized polyclonal rabbit anti-mouse immunoglobulin. The Human Antibody Capture Kit includes a monoclonal mouse anti-human IgG (Fc) antibody capable of capturing all IgG subclasses. The Human Fab Capture Kit features a mix of monoclonal antibodies and capture Fab through kappa and lambda chains. ScFv antibodies may be captured using protein L or variants thereof (22). In cases where a small antibody fragment, antigen or an Fc-receptor is captured to the sensor surface the His Capture Kit can be used to capture histidine-tagged molecules using a monoclonal anti-histidine antibody. Alternatively, biotinylated reagents can be captured onto Biacore Sensor Chip SA, or for a reversible biotin-streptavidin interaction, Biotin CAPture Kit (Fig 4C) can be used.

**Deeper insight into biotherapeutic characteristics with improved efficiency**

Early assessment of expression levels, target specificity, and binding stability is essential for clone selection. The number of samples from hybridoma cells or recombinant expression systems varies greatly but may range up to thousands. A multi-step approach is often applied as shown in Figure 5.

![Construction of clone library](image)

- Yes/No binding
- Ranking, specificity, kinetics
- Developability aspects
- Clones for re-engineering

**Fig 5.** A stepwise approach to antibody selection. The clone library is analyzed to allow selection of candidates for re-engineering.

During these stages, samples are often crude and of limited volume. ELISA based methods are often used in screening and provide end-point results. In contrast, Biacore systems can monitor and quantitate the entire interaction and samples from cell culture supernatants can be injected directly (23). With the assay setup described in Figure 6, injections for capture of antibody and antigen binding are made in sequence (Fig 6A). The antibody capture step provides information related to the expression level/concentration of the antibody and the second injection provides information on the rate and stability of the interaction with antigen. Additionally the antigen binding level may provide stoichiometric information and a closer inspection of the buffer flow phase may reveal whether the interaction is monophasic or biphasic (Fig 6B).

![Screening data](image)

With hundreds to thousands of sensorgrams, it may be convenient to condense the result into plots of report points (Fig 7). In Figure 7A, the position of two regions: stability early and stability late are highlighted in the sensorgrams. In Figure 7B, a plot of the response levels associated with these regions are used to rapidly identify binders with slow off-rate. In this way, Biacore systems are capable of yielding high-content information with single injections of antibody and antigen. Furthermore, data analysis is straightforward and can be focused directly to the desired binding properties.
**Epitope binning for selection of appropriate specificities**

Antigen binding may be the first selection criterion but this has to be complemented with knowledge about the epitope specificity (24) and whether the antibodies are capable of asserting the desired biological effect. Epitope binning experiments serve to identify and bin together antibodies with similar epitope specificity. Epitope binning experiments (25) are easily performed on Biacore systems by testing the selected antibodies against each other in a combinatorial setup. The experimental design includes capture of the first antibody, blocking of free antibody binding sites, injection of antigen, and finally injection of the second antibody.

The assay setup and a corresponding sensorgram are schematically shown in Figures 8A and 8B and the result matrix is based on user-defined thresholds for antibody and antigen binding. When both antibodies can bind, the boxes are blue and a first estimate of the stability of the entire complex is indicated by fitting a dissociation rate constant to the last dissociation phase of the sensorgram.

Yellow boxes indicate that antibodies share the same or have overlapping epitopes. In cases where the binding responses are too low or antigen dissociates too rapidly from the first antibody, mapping information may not be available and this is shown as white boxes. These pairs can be revisited in a second experiment using longer injection times, or if antigen dissociates rapidly, the dual-inject function in Biacore T200 can be used to inject the second antibody directly after the antigen injection with zero dissociation time. In this way it is possible to obtain high-resolution maps. Antibodies with shared or overlapping epitopes may be candidates for therapeutic use provided they exhibit the expected biological effect while antibodies directed towards different epitopes may be candidates for a sandwich assay. To identify epitopes more precisely, inhibition mapping (18) can be performed by pre-incubating the antibody with antigen domains or antigen-derived peptides and observing whether these constructs inhibit antibody binding to the antigen.
**Kinetic analysis for improved understanding of drug activity**

Kinetic data resolves the affinity of an interaction and provides information about the rate of binding and the stability of binding. The affinity constant, $K_D$, is thus separated into an association rate constant, $k_a$, and a dissociation rate constant, $k_d$, where $K_D = k_d / k_a$.

Kinetic data introduces the time domain and allows a better understanding of target occupancy and drug residence time (26, 27). It may also contribute to the understanding of off-target effects. During humanization and re-engineering efforts, it provides detailed information on how structural changes affect binding properties. Engineering efforts may involve antigen sites, Fc-receptor binding sites for improved (28–30) or reduced (31) receptor binding, T-cell epitopes (15), introduction of linker positions for antibody drug conjugates (32), and even more complex tasks if the antibody is to be developed into a bi- or multi-specific antibody (8). Kinetics is also used in developability assays where the effects of forced degradation on binding properties (11, 12, 20) can be studied and where binding to the neonatal Fc-receptor can be used to estimate antibody half-life (16). Thermodynamic analysis has also been suggested as a tool to assess developability and a screening approach to thermodynamics has been described (33) where a favorable ratio of association rate constants determined at 13ºC and 37ºC was indicative of the specificity of binding.

**Kinetic analysis formats for rapid and informative selection of candidates**

Antigen typically binds to antibodies with association rate constants in the range from $5 \times 10^5$ M$^{-1}$ s$^{-1}$ to $5 \times 10^6$ M$^{-1}$ s$^{-1}$ and with dissociation rate constants ranging from $1 \times 10^{-5}$ s$^{-1}$ to $1 \times 10^{-3}$ s$^{-1}$ as illustrated in the $k_a$ vs $k_d$ plot in Figure 9.

![Fig 9. $k_a$ vs $k_d$ plot for typical antibody-antigen interactions. Response curves for conditions A to E are shown in Figure 10.](image)

To illustrate possible outcomes of kinetic experiments, the rate constants associated with points A to E were used to simulate sensorgrams using varying antigen concentrations and varying injection and dissociation times. The antigen was assumed to bind antibody according to a 1:1 interaction model. The resulting scenarios illustrate different screening and characterization approaches (Fig 10).

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![Fig 10. Antibody screening and characterization scenarios. Antigen is injected at various concentrations and for varying time as indicated to the left. Conditions A to E correspond to rate constants defined in Figure 9. The red horizontal line in A to E plots indicates the saturation response. The normalized plots were obtained by re-scaling sensorgrams on a % scale and are intended for comparison of off-rates as indicated by the red ovals.](image)
The top row shows sensorgrams based on a single injection of antigen at 10 nM concentration. Injection and dissociation times were 30 s and the maximum binding level is indicated by the red horizontal line. Binding is clearly observed for conditions B, C, and E but none of the sensorgrams reach the saturation level. The column to the right is based on normalized data and allows a direct comparison of relative dissociation rates. With a 30 s dissociation phase and low binding levels the resolution of off-rates is poor. As the concentration of antigen is increased tenfold to 100 nM binding associated with all conditions (A to E) is readily observed but again the resolution of off-rates is poor. With two injections of antigen at 10 and 100 nM performed in single cycle kinetics mode the response levels for conditions A and D are getting higher and as the dissociation time is now 60 s, the resolution of off-rates is starting to improve. When the experiment is extended to four antigen injections and antigen is injected from 7.7 to 200 nM (3-fold dilutions) for 90 s, binding curves become more suitable for detailed kinetic analysis. Note that with a dissociation phase of 480 s, it is now easy to differentiate between $k_d$s of $10^{-5}$ s$^{-1}$ and $10^{-6}$ s$^{-1}$ and it starts to be possible to differentiate $k_d$s between $10^{-3}$ s$^{-1}$ and $10^{-4}$ s$^{-1}$. By extending injection and dissociation times and by increasing the concentration range more detailed kinetic data is obtained. For practical reasons high-resolution $k_d$ analysis will typically be restricted to a few selected samples. It will take at least 2.9 h to monitor a 10% decrease in the signal level for a dissociation rate constant of $1 \times 10^{-5}$ s$^{-1}$. The corresponding kinetic half-life is 19.3 h.

These scenarios demonstrate how screening with one low antigen concentration can be used to select rapid binders with slow off-rates. Using injection and dissociation times of 30 s will identify slow binders and antibodies from which antigen dissociates rapidly (Fig 7). These antibodies can be deselected. Remaining antibodies can be re-tested using the same antigen concentration injected for a longer time (90 s) and with a longer dissociation time (10 min) to allow better differentiation of off-rates. A few remaining candidates may then be characterized in more detail using a series of antigen concentrations and a further extended dissociation time.

**Early indication of cell- or antibody-mediated cytotoxicity effect**

Fcγ-receptors interact with IgG to regulate the immune response with implications for a number of disease states (34). From a therapeutic antibody perspective, binding of IgG to Fcγ-receptors may be of particular interest for anti-cancer antibodies where FcγIII-receptors on natural killer cells have been implicated in antibody-dependent cell-mediated cytotoxicity (34). IgG binds to Fcγ-receptors with varying affinity and in contrast to antibody-antigen interactions the medium- to high-affinity interactions seen with Fcγ-receptors I and III are often complex and cannot readily be fitted to a simple interaction model (35).

The complexity most likely arises from heterogeneity in glycosylation that is found in both antibodies and receptors. Antibody binding to FcγIII-receptors is thus directly linked to efficacy and it will be important to characterize binding events. Two main approaches to Fcγ-receptor analysis have been established (35, 36). In one approach histidine-tagged Fcγ-receptor is captured by an anti-histidine antibody and antibody is injected (Fig 11a). In the second approach a Protein A surface is used. Antibody is captured and Fcγ-receptor is injected (Fig 11b).

![Fig 11. Antibody- Fcγ-receptor interactions are studied using either histidine-tagged receptor captured on an immobilized anti-histidine antibody (Fig 11A) or antibody captured to protein A (Fig 11B). “A” denotes protein A and “H” denotes a histidine tag. The sensorgrams show the interaction between Rituximab and Fcγ-receptor IIIaVal 158.](image_url)
Both assay types are easy to set up, avoid covalent immobilization of antibody or receptor, and regeneration of sensor surfaces is provided as part of the capture protocols (Fig 5). Note that the two approaches reflect heterogeneity in different ways as the orientation of molecules differ. The shape of the sensorgrams can therefore not be expected to be identical. Kinetic analyses of these interactions are challenging due to pronounced heterogeneity [35], therefore an alternative approach to data analysis based on a statistical comparison of sensorgrams has been suggested [37] for use in comparability studies.

**Better prediction of candidate efficacy**

Antibody clearance and half-life is partly determined by its interactions with the neonatal Fc-receptor, FcRn. Antibodies that are taken up into the endosome and directed for degradation in lysosomes can be salvaged from degradation and recirculated by binding to FcRn. Binding to FcRn in the endosome takes place at pH 6.0 and the antibody is released at pH 7.4 when it is returned to the cell surface [38]. Increased half-life is of interest from an efficacy and dosage perspective and part of antibody engineering projects.

The pH-dependence of antibody-FcRn interactions is easily studied in Biacore T200 (Fig 12). Biotinylated FcRn is captured to streptavidin using the Biotin CAPture Kit. The running buffer is maintained at pH 6.0. Antibody is injected at pH 6.0 using the dual inject function. It binds to the receptor and when the antibody injection stops it is immediately followed by injection of buffer at pH 7.4 to monitor dissociation at this pH. With this procedure it is possible to seamlessly establish relevant conditions for both binding and release of antibody from FcRn. Note that the solutions for “dual inject” can be selected freely and this inject type can therefore be used to introduce new buffer conditions while the running buffer remains the same.

The active concentration of a protein is defined through the interaction of the protein with its binding partner and can differ from the total protein concentration if some of the molecules are incapable of binding. If a protein has several different binding sites an active concentration can be established for each binding site. Biacore T200 system can be used to directly determine the concentration of an analyte provided that binding rates are flow rate-dependent. This type of analysis is called calibration-free concentration analysis (CFCA) because the concentration is determined directly without the use of an external standard. From a practical perspective, CFCA is possible when binding rates increase with increasing flow rate (Fig 13A). Relative CFCA data for three biotherapeutic medicines: bevacizumab, rituximab, and interferon α-2a are listed in Figure 13B.

![Fig 12. Binding of infliximab to hFcRn.](image)

**CFCA for assessment of antibody concentration and integrity**

The CFCA data obtained were generally in good agreement with expected values because it ranged from 82% to 88% of the labeled concentration. This demonstrates that reasonable concentration values can be obtained using CFCA. Remaining uncertainties in the fitting assumptions may explain the difference but there may also be some uncertainty in the labeled value.

![Fig 13.](image)
With these promising results, potential application areas of CFCA include:

- Comparison to concentration data obtained by absorbance measurements to verify that a reliable concentration value is used in affinity and kinetic analysis (39).
- Use of CFCA to identify changes in binding properties that occur in forced degradation studies.
- Developability assessments of bispecific or multi-specific antibodies where relative CFCA measurements can be used to test the integrity of these constructs.

The use of CFCA as a potency assay is further discussed (40). Additionally, Biacore systems support concentration measurements using standard curves. Regular concentration measurements show wide dynamic range and very good precision (40).

**Conclusion**

As early and late stage antibody development processes become more integrated (40), analytical technologies that guide developers and support decision making become key tools in these workflows. Biacore systems deliver the resolution, sensitivity, precision, throughput, ease-of-use, and assay versatility required to develop a first candidate into a clinical lead with the critical quality binding attributes required for approved biologics.

**References**
